Profiling gene expression in whole blood samples following an in-vitro challenge

Spijker, S.; van de Leemput, J.C.H.; Hoekstra, C.; Boomsma, D.I.; Smit, A.B.

published in
Twin Research
2004

DOI (link to publisher)
10.1375/1369052042663878

document version
Publisher's PDF, also known as Version of record

Profiling Gene Expression in Whole Blood Samples Following an In-Vitro Challenge

Sabine Spijker,[1] Joyce C. H. van de Leemput,[1] Chantal Hoekstra,[2] Dorret I. Boomsma,[2] and August B. Smit[1]

[1]*Department of Molecular and Cellular Neurobiology, Vrije Universiteit, Amsterdam, the Netherlands*
[2]*Department of Biological Psychology, Vrije Universiteit, Amsterdam, the Netherlands*

Genomics tools (gene- and protein-expression studies) can be used to find possible target genes involved in a quantifiable trait or disease state. However in many instances, cells and tissues directly involved in the trait's expression, for example, brain tissue, are not amenable for gene expression analysis. Whole blood cells share a molecular make-up for cellular communication and gene regulation systems with many other cell types, for example, neuronal cells, and have the advantage of being very accessible for gene profiling. We investigated the feasibility of nationwide blood sample collection for lymphocyte RNA isolation and real-time PCR analysis to quantify genomic responses. We tested several designs for blood collection and storage: blood sampling in PAXgene blood collection tubes and storage at –20°C, blood sampling in heparin tubes and decanting the samples (with or without in-vitro stimulus) into either PAXgene blood collection tubes and storage at –20°C, or polypropylene tubes followed by snap-freezing and storage at –80°C. The latter procedure is the best cost-wise when only small amounts of total RNA are needed for downstream applications. Lymphocyte gene expression studies are most likely hampered by the quality of isolated RNA rather than the sampling method. We show that large-scale nationwide sample collections did not alter RNA quality or gene expression levels when compared to sampling and processing in a more controlled way. To this end, we present an optimized protocol for easy and standardized isolation of high quality RNA using the PAXgene isolation kit. Based on these results, we suggest that whole blood genomic data can be used as a genomic probe in experimental and clinical research.

Gene expression is controlled by a constellation of so-called transcription factors that are part of a genetic network. The expression of genes in turn is determined by genetic make-up, that is, by individual-specific sequences in the regulatory elements of these genes, and by the cellular environment that influences transcription. Gene expression therefore carries both valuable information on the individual's genome, as well as on the individual's genome–environment interaction. Assessing genome expression in the human context, however, is often unrealistic since biopsies of certain tissues from living patients cannot be obtained. To this end, blood lymphocytes have been proposed as a convenient and accessible alternative (Gladkevich et al., 2004). Blood cells share a molecular make-up for cellular communication and basic gene regulation systems with other types of cells in the body (Blalock, 1994). In principle, blood cells carry information on a subset of the genetic network relating to the genes expressed in these cells. As such they might pinpoint genes of which the control is affected by mutation (e.g., polymorphisms). In addition, blood cells form biosensors of which the gene expression is influenced by the surrounding body fluid and all molecules therein. As such, gene expression of blood cells might reveal previous or developing disease states and thus present valuable diagnostic markers of an individual.

In addition, blood cells have the advantage that various stimuli, or challenges, can easily be applied to elicit genomic responses that may teach us more about deficiencies in the underlying genetic network. Indeed, studies on blood lymphocytes have revealed a close association between the state of the immune system and major psychiatric disorders (Coplan et al., 1999; Schwarz et al., 2001; Stastny et al., 2003). In this study, whole blood samples were stimulated with dexamethasone as glucocorticoids have pronounced effects on the metabolism, differentiation, proliferation, and cell survival of both the peripheral and central systems (Alarid et al., 2003; Bianchi et al., 2000; Rowland et al., 1998; Tonko et al., 2001).

Gene expression studies on stimulated whole blood samples might include large-scale open (microarray) or medium-scale targeted (real-time PCR) screenings. Microarrays are a high-throughput system where the expression of thousands of genes can be analyzed at the same time, whereas real-time

Received 28 July, 2004; accepted 2 October, 2004.

Address for correspondence: Dr. Sabine Spijker, Dept. Molecular and Cellular Neurobiology, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, the Netherlands. E-mail: sabine.spijker@falw.vu.nl

Table1

Comparison of RNA Yield (Standardized from 2.5 ml Blood) Using Different Procedures

	Average yield (*μg/μl*) ± *SD*	*n*
PAX direct	5.8 ± 1.3	7
Frozen in PAX	8.7 ± 2.0	24
Frozen before PAX	3.7 ± 1.7	60

Note: Blood can be drawn immediately into the PAXgene collection tube (*PAX direct*), or can be drawn into heparin-coated tubes, whereafter the blood sample is either directly transferred to the PAX tube (*frozen in PAX*), or it followed the procedure as described in this paper (*frozen before PAX*).

n indicates the number of isolations on which this observation was based.

PCR is more labor-intensive. Real-time PCR is much more sensitive both to low abundant RNA species (transcription factors, receptors, transporters) as well as to subtle changes in gene expression (Spijker et al., 2004). Microarrays have previously been applied to study gene expression changes in whole blood samples resulting from various types of stimuli (Spijker et al., unpublished results). In this study, a limited number of genes were selected and real-time PCR used to quantify the genomic responses. In addition, the feasibility of nationwide blood sample collection of mothers of dizygotic twins (MODZT) for which the blood samples were challenged with dexamethasone during transport was tested by comparing it to blood samples from local controls (colleagues) that were sampled and processed in a more controlled way. From these samples, the quantity and integrity of isolated RNA was checked, and gene expression measured under basal and dexamethasone-induced conditions. Moreover, different blood collection and storage procedures were compared.

Materials and Methods

Sample Collection

For testing different procedures of blood collection and storage (Table 1), serial venous blood samples (maximally four 9 ml tubes within 4 minutes) were taken from 11 healthy female donors (controls) after informed consent using safety-lock butterfly needles. An in-vitro stimulus was applied to some of these samples (see below). Blood from only six of the female donors (average age 31.8 ± 6.0), who were sampled at the same time (all samples within a period of 10 minutes), was used for measuring gene expression, and one donor was sampled twice with a 6-month delay. Blood was drawn between 11:00 a.m. and 1:00 p.m. For the six controls used for gene expression measurements, blood was drawn between 12:30 p.m. and 1:00 p.m. Blood was collected either directly into PAX blood collection tubes and stored for a maximum of 2 months at –20°C (*PAX direct*, Table 1), or it was collected into heparin-coated tubes (Greiner) and (with or without various in-vitro challenges) decanted into either PAX blood collection tubes and stored for a maximum of 2 months at –20°C (*frozen in PAX*; Table 1), or polypropylene tubes after which the samples were snap-frozen on a mixture of dry ice and ethanol (*frozen before PAX*; Table 1). For the latter procedure, blood was thawed into PAX blood collection tubes after which it followed the standard procedure of RNA isolation (described below). All blood collection tubes were inverted at least eight times immediately after collection and before the next processing step.

Serial venous whole blood samples from three mothers of dizygotic twins (MODZT) donors (see Hoekstra et al., 2004) were obtained after informed consent using safety-lock butterfly needles (within 6 minutes). Sampling took place during a two-month period, with blood drawn between 7:00 a.m. and 10:00 a.m. Two additional EDTA tubes were drawn before blood sampling for RNA isolation (see Hoekstra et al., 2004). Samples were collected after overnight fasting in two 9 ml lithium/sodium heparin coated tubes (Greiner), and were decanted (with or without in-vitro challenges) into polypropylene (storage) tubes, after which they were snap-frozen (see below). All blood collection tubes were inverted at least eight times immediately after collection and before the next processing step.

Challenge Application and Storage

Before proceeding to the next step, (either direct freezing or application of a stimulus; see below), all samples were transferred and/or kept at room temperature. For MODZT samples, proceeding to the next step took for the most part between 10 and 30 minutes after sampling (but no longer than one hour). For control samples, this was within 20 minutes after sampling. Whole blood samples (MODZT and controls) collected in one of the heparin tubes from each participant were divided into three ~3 ml portions in 4 ml polypropylene tubes (Greiner). These aliquots were snap-frozen, and stored at –80°C until further processing. A challenge was applied to the second heparin tube by stimulation with 10^{-7} M Dexamethasone (Dex; Sigma). For controls, a third (*E. coli* LPS, [Sigma] 10 ng/ml blood) and fourth (10^{-7} M Dexamethasone and *E. coli* LPS, 10 ng/ml blood) heparin tube were stimulated as well. All stimulated samples were incubated at 37°C. For controls, samples were rotated in a room of constant temperature, and for MODZT, the challenge was induced during transport and continued after arrival at the Leiden laboratory for no longer than 6 hours (variations of 30 minutes). The latter samples lay flat in the insulated box of constant temperature (37°C) and were mixed by the motion of the transporting vehicle. After all stimulations, sample tubes were inverted at least eight times and subsequently divided in three ~3 ml aliquots, snap frozen and stored at –80°C.

RNA Isolation

After storage of the MODZT and control samples at –80°C, the tubes containing the ~3 ml aliquots of whole blood were thawed in PAX (PreAnalytiX; Qiagen) gene

blood collection tubes (added volume of 2.5 ml blood; maximum capacity of the PAX tubes), inverted at least 10 times and incubated at room temperature for at least 3 hours. PAX gene blood collection tubes with blood samples (*PAX direct* and *frozen in PAX*; Table 1) stored at –20°C were thawed at room temperature for at least 2 hours, and were inverted at least three times before further processing. Total RNA was extracted according to the manufacturer's protocol (Qiagen) with several important adjustments. To ensure full separation and subsequent removal of all protein debris, 325 µl BR2 binding buffer (instead of 300 µl) was added to the BR1 buffer prior to proteinase K-treatment, and after 10 minutes of incubation at 50°C and 1 minute of centrifugation at 14,000 rpm, debris was removed by sucking the debris into a 1 ml tip while the supernatant was kept for further processing. To ensure complete genomic DNA removal for the downstream real-time PCR application, an on-column DNase digestion was performed with the Qiagen RNase-free DNase set (Qiagen). Furthermore, a total of 90 µl BR5 elution buffer (twice 45 µl) was used to elute the sample from the column.

The quantity and integrity of the extracted RNA was measured using the nano-assay on the Agilent 2100 Biosizing (Agilent Technologies). In addition, quantity of extracted RNA was determined using spectrophotometry (NanoDrop ND–1000 UV-Vis Spectrophotometer, Nanodrop Technologies). Typical RNA yields after storage at –80°C is 2–4 µg per PAX isolation, whereas this was 4–6 µg when whole blood samples were processed directly into PAX-tubes after heparin collection or DEX-challenge. RNA samples were then stored at –80°C until further processing.

Reverse Transcription

For downstream real-time PCR analysis, 500 ng of total RNA was reverse transcribed using random primers (25 pmol), RNA-guard (15 U, Promega) and MMLV reverse transcriptase (20 U; Roche) according to the manufacturer's protocol in a volume of 25 µl for 1 hour at 37°C. Following synthesis, samples were precipitated by addition of 3 volumes of 100% ethanol and 0.1 volume of NaAc (3 M, pH 5.6), incubation on ice for 30 minutes and centrifugation for 30 minutes at 14,000 rpm at 4°C. The supernatants were discarded and pellets were washed by addition of 500 µl 70% ethanol and centrifugation for 5 minutes at 14,000 rpm at 4°C. The supernatants were then thoroughly removed, and the pellets air-dried (approximately 10 minutes at 37°C). Then, pellets were resuspended in 25 µl distilled water and samples were stored at 4°C.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

For measuring relative expression levels, PCR reactions (10 µl; ABI PRISM 7900) were performed with transcript-specific primers (600 nM) on cDNA corresponding to ~2 ng RNA, using cycle-settings: 10 minutes 50°C, 40 cycles of 95°C for 20 seconds, 60°C for 1 minute, followed by a dissociation from 60°C to 95°C in 15 minutes. Criteria for and design of primers as well as calculations were as described previously (Jacobs et al., 2002). In short, three potential reference genes (encoding hypoxanthine phosphoribosyl transferase [HPRT], glyceraldehyde 3-dehydrogenase [GAPDH] and β-actin) were measured first for stable relative expression across all individual samples. From this, two genes (encoding GAPDH and β-actin) were selected as reference genes. Then for each gene (encoding DAF, DUSP6, NFKBIA, TNFAIP3, RGS2, TOB1 and VDUP) the cycle of threshold (Ct) values were used to calculate the relative level of gene expression normalized to the mean of the two reference controls in each sample (HEP, expression in the heparin sample; DEX, expression in the Dex-stimulated sample) which will be denoted by Ct_{norm}. For each sample, the Dex-induced expression, indicated as the Dex-induced ratio (log_2-scale) for gene x, r_x, was calculated from the normalized values as

$$r_x = (Ct_{normx,\,HEP} - Ct_{normx,\,DEX})$$

and average regulation values were calculated from these individual ratios.

Results and Discussion

RNA Yield and Purification

Integrity of RNA samples is essential for any downstream application, for example, real-time PCR or microarrays. Isolation of RNA from whole blood samples using the PAX system yielded high quality RNA where both ribosomal bands were visible (28S more abundant than 18S, Figure 1). There was no difference in RNA quantity or quality for heparin or Dex-stimulated samples. However, a difference in RNA quantity was observed after freezing samples. There was a ~30% reduction in RNA yield when compared to blood sampling directly into PAX tubes, as suggested by the manufacturer. Despite this reduction, freezing whole blood samples prior to adding them to the PAX-buffer is a good alternative for large longitudinal studies where gene expression analysis needs little RNA (real-time PCR or microarrays with amplified RNA). The highest RNA yield was obtained when blood was first drawn into heparin tubes, transferred to the PAX tube (Table 1) and frozen at –20°C. There were no differences between yields for individuals, nor for the stimulus applied (ANOVA). As an additional control, gene expression was measured from blood samples of one control sampled twice in a period of 6 months.

A critical step in the RNA isolation procedure using the PAX-system is the removal of proteins from the sample prior to loading it onto the RNA isolation column. After addition of buffer BR2 (typically add 325 µl) and the proteinase K-treatment, proteins should aggregate and be removed from the sample. If this aggregate does not form, additional buffer BR2 should be added.

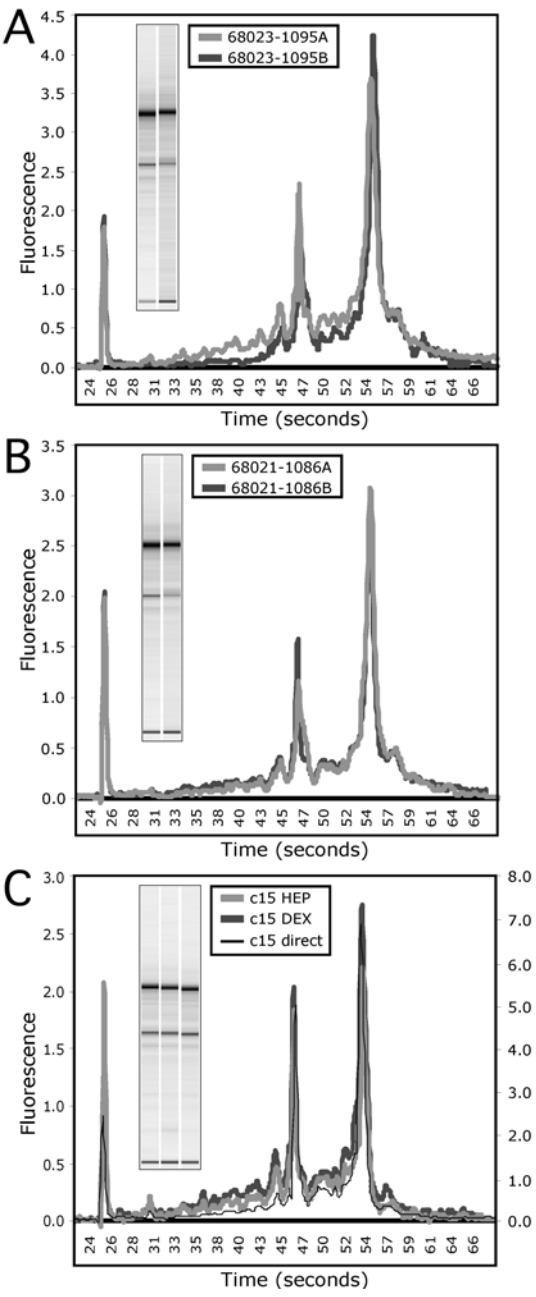

Figure 1

Integrity of total RNA from whole blood samples before and after DEX stimulation.

Electropherograms (and gel-like images, inset) are shown for RNA isolations from two MODZT donors (68023–1095, A; 68021–1086, B) and a control donor (c15, C). The ribosomal RNAs (18S and 28S, 46–48 minutes and 52–58 minutes, respectively) can be distinguished as two sharp peaks with ratios 28S/18S approaching 2, in addition to the marker peak (25 minutes), which allows for sample alignment. There was no difference in RNA quality due to the Dex-stimulation (compare electropherograms of heparin samples [samples 'A' in panels A and B and 'HEP' in panel C] to those after Dex-stimulation [samples 'B' in panels A and B; 'DEX' in panel C]. RNA isolation of whole blood drawn into a PAX-tube directly (C, 'direct', thin black line; y-axis on the right) showed that freezing/thawing whole blood samples prior to addition of the PAX isolation buffer had no effect on the quality of isolated RNA, but only had an effect on the quantity of isolated RNA. Note the absence of a 5S ribosomal peak in all electropherograms due to silica-column isolation.

Many gene expression studies of blood samples involve isolation of a particular cell population from whole blood. Unpublished studies in our laboratory showed a dramatic change in basal gene expression of whole blood sample after centrifugation. To this end, using only whole blood samples in combination with the PAX-isolation method for RNA is suggested when looking at basal gene expression.

Gene Expression in Peripheral Blood Samples

Previously we performed microarray experiments with RNA extracted from whole blood samples stimulated with Dex or without stimulation (unpublished results). Based on these experiments, several genes were selected that were abundantly expressed in peripheral blood cells and/or were regulated by Dex. These were used for a SYBR Green dye-based real-time PCR analysis. There are two house-keeping genes among these that served as internal reference for normalization of input-RNA. Our real-time PCR used two conventional primers to amplify cDNA and the SYBR Green I dye, a highly specific double-stranded DNA binding dye, for detection of formed product during PCR. In addition, this assay has the advantage of being highly sensitive and allows post-hoc analysis of the specificity of the formed PCR product.

Firstly, the expression level of several potential reference genes (β-actin, HPRT and GAPDH) was measured across all individual samples for stable relative expression (Garcia-Vallejo et al., 2004). This has to be established for each cell-type used as (1) the mRNA levels of the target genes are expressed in a relative way to the reference genes, and (2) it is known that some of the potential reference genes are differentially expressed due to cell-type or cell-treatment (Hamalainen et al., 2001). As a measurement for this, the *SD* was calculated on the relative expression of each set of two potential reference genes in each sample. The lowest *SD* was obtained for the GAPDH/β-actin combination. We observed an *SD* of 0.46, which is reasonably stable when compared to the *SD* values obtained from brain tissue of individual isogenic laboratory animals (~0.18).

Gene expression for all heparin samples was analyzed to verify the quality of cDNA (Figure 2). Gene expression for all transcripts in MODZT donors ($n = 3$) was very similar to that obtained from controls ($n = 6$). A reason for the low variation observed in basal expression levels (Whitney et al., 2003) could be that gene expression was measured with real-time PCR. However, a striking difference was observed for the expression of RGS-2: RGS-2 has reduced expression in all MODZT donors ($p < .005$; t test; Figure 2A, B). Although subtle differences in sample processing could explain this difference in basal expression level (albeit that other genes are not affected by this), it may be also be caused by other factors such as genetic predisposition and limited sample size. Future study will shed more light on this.

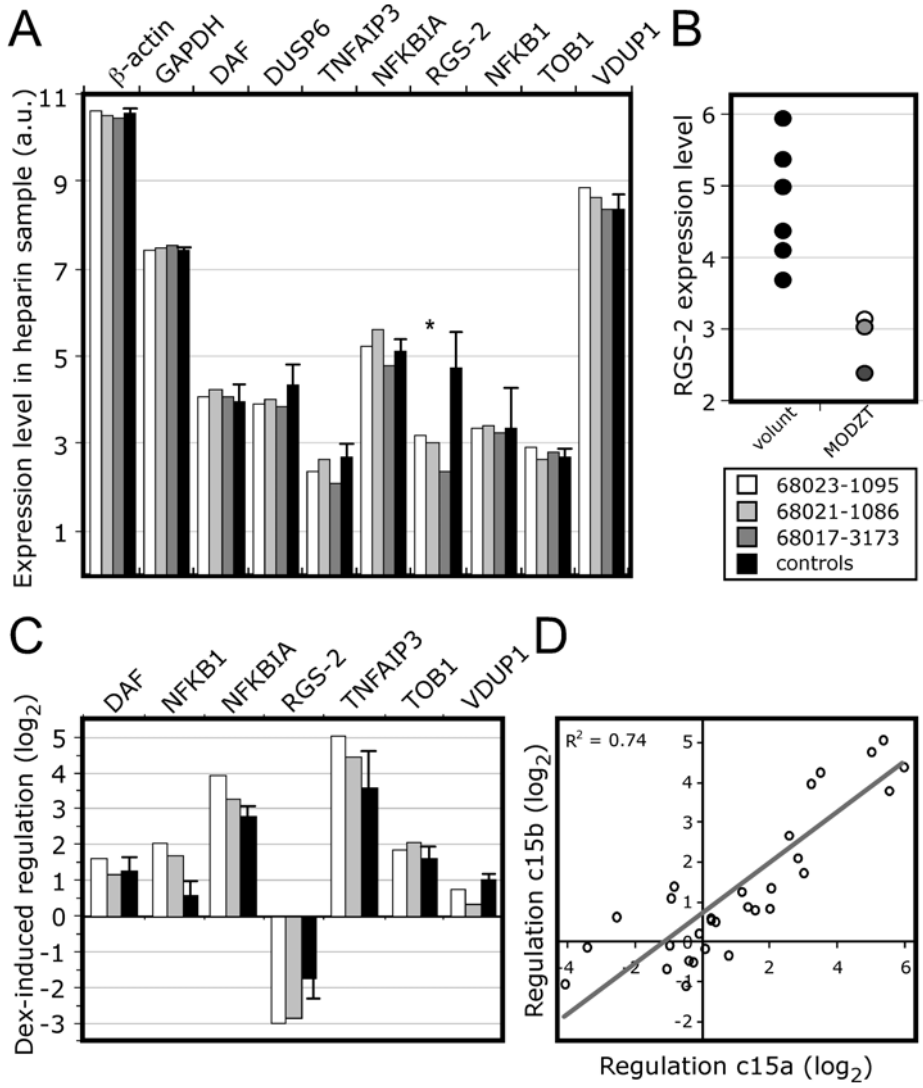

Figure 2

Gene expression in whole blood samples using qPCR.

Gene expression was measured for several genes abundantly expressed in whole blood samples. A) Relative expression level (normalized to the average of the control genes) of several genes (DAF: complement decay-accelerating factor/CD55; DUSP6: dual specificity protein phosphatase 6/MKP3; TNFAIP3: tumor necrosis factor, alpha-induced protein 3; NFKBIA: NF-kappaB inhibitor alpha/MAD3; RGS-2: regulator of G-protein signaling 2; NFKB1: Nuclear factor NF-kappa-B p105 subunit; TOB1: Transducer of erbB-2 1; VDUP1: 1,25-dihydroxyvitamin D-3 upregulated protein1/TXNIP) is presented in arbitrary units (log_2-scale) for the average of the control samples (controls, black), and for three individual MODZT donors (white, light gray, dark gray, see legend box). A & B) All genes, except for RGS-2, showed a similar level of expression in the MODZT donors compared with the controls. RGS-2 showed a significant lower expression ($p < .005$; Student's *t* test). C) Dex-induced regulation was measured for a selection of genes, and is presented as fold-regulation (to heparin; log_2-scale) for the average of the control samples, and for two individual MODZT donors. For all genes, the Dex-induced regulation was significant ($p < .01$; Student's *t* test) Dex-treatment resulted in similar activation of genes. In the MODZT samples, RGS-2 and TNFKB1 show slightly more repression and induction respectively. D) Dex-, LPS- or Dex+LPS-induced regulations for several genes of samples (a and b) taken from one control (c15) within a 6 month timeframe were compared to each other. The line indicates the correlation, the R^2 value is indicated.

Next, Dex-induced regulation was analyzed in MODZT samples ($n = 2$), and controls ($n = 6$) for a selected set of genes. Expression of several genes is known to be affected by Dex-stimulation in various cell types, that is, NFKBIA (Alcorn et al., 2004; Chauhan et al., 2002; Medh et al., 2003), TNFAIP3 (Galon et al., 2002), RGS2 (Homme et al., 2003), and VDUP (Medh et al., 2003). All selected genes were significantly induced (Student's *t* test; $p < .01$) upon Dex-stimulation (Figure 2C) in the control samples. It must be noted that increased power was obtained from the paired type of analysis that was performed, that is, normalizing the Dex-induced expression to the heparin-sample of the same individual. MODZT donors showed a similar Dex-induced change in expression for all genes compared with controls. Slightly increased (NFKB1) and decreased (RGS2) expression was observed in the MODZT samples.

Every individual sample was then correlated for all the gene expression values. The averaged correlations of Dex-induced regulations among controls were very similar to the averaged correlations between MODZT and controls (R^2 is .76 and .72, respectively). Based on this, we concluded that although sampling of blood and incubation with the stimulus (Dex) was slightly different for MODZT and controls, small variations in sampling protocols — as is inevitable when sampling from humans or when comparing different studies — could be ignored.

The induced gene expression levels of one control, who was sampled twice within a 6-month timeframe, was compared to analyze the stability of gene expression over time (Figure 2D). The correlation (R^2 is .74) was similar to the averaged correlation value obtained within individuals. In addition to the observation that the RNA quality was good for both samples, the small variation in expression levels for this subject is most likely due to time of blood collection. More observations are needed to exclude any seasonal effect.

Conclusion

It was shown that nationwide blood sampling is feasible in terms of quality of RNA for downstream genomic applications. There is minimal variation between samples when our blood sampling and processing (stimulus incubation, freezing samples prior to RNA isolation) is used. This shows that there are little caveats in the collection procedure in terms of feasibility (transport, etc.) or quality of RNA. This is important because it is inevitable that variation in a collection procedure will exist, even within one study. In particular, our procedure of blood collection and storage (freezing before isolation) is a good alternative for large longitudinal studies where only a limited number of samples will finally be analyzed, as a result of the reduction in chemical and storage capacity costs as well as increased processing efficiency.

Variation in samples as apparent in gene expression studies (apart from biological source variation) could most likely be attributed to the quality of the RNA. It was shown that high quality RNA can be obtained using a simple, easy, and, above all, standardized RNA isolation protocol, which results in reproducible gene expression measurements. Therefore, gene expression analysis on stimulated peripheral blood cells is a feasible tool to screen for biomarkers for a particular trait.

References

Alarid, E. T., Preisler-Mashek, M. T., & Solodin, N. M. (2003). Thyroid hormone is an inhibitor of estrogen-induced degradation of estrogen receptor-alpha protein: Estrogen-dependent proteolysis is not essential for receptor transactivation function in the pituitary. *Endocrinology, 144,* 3469–3476.

Alcorn, J. L., Islam, K. N., Young, P. P., & Mendelson, C. R. (2004). Glucocorticoid inhibition of SP-A gene expression in lung type II cells is mediated via the TTF-1-binding element. *American Journal of Physiology. Lung Cellular and Molecular Physiology, 286*, L767–776.

Bianchi, M., Meng, C., & Ivashkiv, L. B. (2000). Inhibition of IL-2-induced Jak-STAT signaling by glucocorticoids. *Proceedings of the National Academy of Sciences of the United States of America, 97,* 9573–9578.

Blalock, J. E. (1994). Shared ligands and receptors as a molecular mechanism for communication between the immune and neuroendocrine systems. *Annals of the New York Academy of Sciences, 741,* 292–298.

Chauhan, D., Auclair, D., Robinson, E. K., Hideshima, T., Li, G., Podar, K., Gupta, D., Richardson, P., Schlossman, R. L., Krett, N., Chen, L. B., Munshi, N. C., & Anderson, K. C. (2002). Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays. *Oncogene, 21,* 1346–1358.

Coplan, J. D., Tamir, H., Calaprice, D., DeJesus, M., de la Nuez, M., Pine, D., Papp, L. A., Klein, D. F., & Gorman, J. M. (1999). Plasma anti-serotonin and serotonin anti-idiotypic antibodies are elevated in panic disorder. *Neuropsychopharmacology, 20,* 386–391.

Galon, J., Franchimont, D., Hiroi, N., Frey, G., Boettner, A., Ehrhart-Bornstein, M., O'Shea, J. J., Chrousos, G. P., & Bornstein, S. R. (2002). Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. *The Faseb Journal, 16,* 61–71.

Garcia-Vallejo, J. J., Van Het Hof, B., Robben, J., Van Wijk, J. A., Van Die, I., Joziasse, D. H., & Van Dijk, W. (2004). Approach for defining endogenous reference genes in gene expression experiments. *Analytical Biochemistry, 329*, 293–299.

Gladkevich, A., Kauffman, H. F., & Korf, J. (2004). Lymphocytes as a neural probe: Potential for studying psychiatric disorders. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 28,* 559–576.

Hamalainen, H. K., Tubman, J. C., Vikman, S., Kyrola, T., Ylikoski, E., Warrington, J. A., & Lahesmaa, R. (2001). Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Analytical Biochemistry, 299,* 63–70.

Hoekstra, C., Meijer, P., Kluft, C., Heutink, P., Smit, G., de Geus, E., Smit, J. H., van Bruggen, A., Montgomery, G. W., & Boomsma, D. I. (2004). Genetics of dizygotic twinning. A feasability study for a biobank. *Twin Research, 7,* 556–563.

Homme, M., Schmitt, C. P., Himmele, R., Hoffmann, G. F., Mehls, O., & Schaefer, F. (2003). Vitamin D and dexamethasone inversely regulate parathyroid hormone-induced regulator of G protein signaling-2

expression in osteoblast-like cells. *Endocrinology, 144*, 2496–2504.

Jacobs, E. H., Spijker, S., Verhoog, C. W., Kamprath, K., de Vries, T. J., Smit, A. B., & Schoffelmeer, A. N. (2002). Active heroin administration induces specific genomic responses in the nucleus accumbens shell. *The Faseb Journal*, *16,* 1961–1963.

Medh, R. D., Webb, M. S., Miller, A. L., Johnson, B. H., Fofanov, Y., Li, T., Wood, T. G., Luxon, B. A., & Thompson, E. B. (2003). Gene expression profile of human lymphoid CEM cells sensitive and resistant to glucocorticoid-evoked apoptosis. *Genomics, 81,* 543–555.

Rowland, T. L., McHugh, S. M., Deighton, J., Dearman, R. J., Ewan, P. W., & Kimber, I. (1998). Differential regulation by thalidomide and dexamethasone of cytokine expression in human peripheral blood mononuclear cells. *Immunopharmacology, 40*, 11–20.

Schwarz, M. J., Muller, N., Riedel, M., & Ackenheil, M. (2001). The Th2-hypothesis of schizophrenia: A strategy to identify a subgroup of schizophrenia caused by immune mechanisms. *Medical Hypotheses, 56*, 483–486.

Spijker, S., Houtzager, S. W., De Gunst, M. C., De Boer, W. P., Schoffelmeer, A. N., & Smit, A. B. (2004). Morphine exposure and abstinence define specific stages of gene expression in the rat nucleus accumbens. *The Faseb Journal, 18,* 848–850.

Stastny, J., Konstantinidis, A., Schwarz, M. J., Rosenthal, N. E., Vitouch, O., Kasper, S., & Neumeister, A. (2003). Effects of tryptophan depletion and catecholamine depletion on immune parameters in patients with seasonal affective disorder in remission with light therapy. *Biological Psychiatry, 53,* 332–337.

Tonko, M., Ausserlechner, M. J., Bernhard, D., Helmberg, A., & Kofler, R. (2001). Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. *The Faseb Journal, 15,* 693–699.

Whitney, A. R., Diehn, M., Popper, S. J., Alizadeh, A. A., Boldrick, J. C., Relman, D. A., & Brown, P. O. (2003). Individuality and variation in gene expression patterns in human blood. *Proceedings of the National Academy of Sciences of the United States of America, 100,* 1896–1901.